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A collaborative EDNAP exercise on SNaPshot™-based mtDNA control region typing

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Abstract

A collaborative European DNA Profiling (EDNAP) Group exercise was undertaken to assess the performance of an earlier described SNaPshot™-based screening assay (denoted mini-mtSNaPshot) [1] that targets 18 single nucleotide polymorphism (SNP) positions in the mitochondrial (mt) DNA control region and allows for discrimination of major European mtDNA haplogroups. Besides the organising laboratory, 14 forensic genetics laboratories were involved in the analysis of 13 samples, which were centrally prepared and thoroughly tested prior to shipment. The samples had a variable complexity and comprised straightforward single-source samples, samples with dropout or altered peak sizing, a point heteroplasmy and two-component mixtures resulting in one to five bi-allelic calls. The overall success rate in obtaining useful results was high (97.6%) given that some of the participating laboratories had no previous experience with the typing technology and/or mtDNA analysis. The majority of the participants proceeded to haplotype inference to assess the feasibility of assigning a haplogroup and checking phylogenetic consistency when only 18 SNPs are typed. To mimic casework procedures, the participants compared the SNP typing data of all 13 samples to a set of eight mtDNA reference profiles that were described according to standard nomenclature [2], and indicated whether these references matched each sample or not. Incorrect scorings were obtained for 2% of the comparisons and derived from a subset of the participants, indicating a need for training and guidelines regarding mini-mtSNaPshot data interpretation.

1. Introduction

Analysis of mitochondrial DNA (mtDNA) has become a routine technique in many laboratories involved in forensic testing and kinship analysis especially when nuclear DNA (nDNA) is severely degraded or absent [3-5]. In its current practice, mtDNA typing typically involves Sanger sequencing of the control region, which contains considerable sequence variation [3,6-9]. In the (near) future, massively parallel sequencing (MPS) may be applied to sequence mtDNA control regions or full mitogenomes. For both sequencing approaches it may be opportune to select interesting samples, especially when a case involves a large sample set. Such pre-assessment may be achieved through single-base extension (SBE) approaches as these examine a selected subset of single nucleotide polymorphisms (SNPs) relatively fast [10-12]. Mutations at positions not included in such an assay will pass unnoticed, so the discriminatory power of the assay will depend on the number and identity of the selected SNP positions.

Recently, we described a SNaPshot™-based mtDNA selection tool [1] that targets 18 SNPs in the mtDNA control region. SNPs were selected for their relative frequency in a European population. This mini-mtSNaPshot assay consists of two SNaPshot™ multiplexes that

pair to the two mini-mtDNA amplification multiplexes that were specifically developed to enable mtDNA sequencing analysis of degraded samples [13]. Due to this pairing, no additional DNA extract is required for sequencing, which is forensically advantageous. Degenerate bases were included in the 3' part of the SBE primers to reduce allele dropout from sequence variation at the primer binding sites. Using degenerate bases in a SNaPshot™ assay can affect extension product sizing and peak morphology. Not all polymorphic sites at the primer binding sites are covered by degenerate bases, and in particular cases signals may be reduced or absent. These effects are explained in Ref. [1].

A collaborative exercise was organised by the Netherlands Forensic Institute (NFI) in order to assess the value of this mini-mtSNaPshot selection tool. Fifteen laboratories (including the organising laboratory) participated in the exercise, some of which had little to no experience with mtDNA or SNaPshot™ analysis. Each participant was provided with centrally prepared primer mixes and the same set of PCR products that represent samples with complexities like allele dropout, altered peak sizing or peak morphology, heteroplasmy and mixed samples with bi-allelic calls. All participants were asked to perform post-PCR clean-up, mini-mtSNaPshot assays and electrophoretic analysis. Participants could perform a phylogenetic haplogroup inference. To mimic casework analyses, participants were requested to compare a set of eight reference profiles given, in standard nomenclature, to the SNP profiles of the samples and assess the possibility of a match. Also at the interpretational level complicating factors were included such as a purposeful mix-up so that multiplex set one and multiplex set two did not originate from the same donor. Therefore, the aim of this exercise included not only the technical implementation of this assay into laboratory practice, but also the meaningful assignment of haplogroups and the interpretation of results in a forensic context.

2. Material and methods

2.1 Samples and materials provided

The mtSNP exercise was divided into two parts: part 1 consisted of laboratory analysis of 13 samples and one blank with the two mini-mtSNaPshot multiplexes and inference of the haplogroup and part 2 comprised a paper challenge comparing the mini-mtSNaPshot data of all 13 samples to a set of eight mtDNA reference profiles that were described according to standard nomenclature [2].

DNA extracts and PCR products were prepared at the organising laboratory with informed consent of the volunteers whose cellular material was used. DNA was extracted using the QIAamp DNA Mini Kit (QIAgen, Venlo, The Netherlands) according to the manufacturer's instructions; the control region was amplified as described in Ref. [1] and ExoSap-IT®

(Affymetrix) treatment was performed according to manufacturer's instructions. The amplified products that are to be analysed with mini-mtSNaPshot multiplex 1 were ExoSap-IT®-treated at the organising laboratory; those to be analysed with multiplex 2 were ExoSap-IT®-treated by the participants. Every participating laboratory received 26 PCR products: 13 for multiplex 1 and 13 for multiplex 2. Also, aliquots of the two SBE primer mixes [1] originating from a single batch of primer mix preparation were provided. ExoSap-IT®, SNaPshot™ Multiplex Ready Reaction Mix and Shrimp Alkaline Phosphatase (SAP, Affymetrix) were provided to one participant. All reagents and samples were shipped on dry ice and arrived in 1 to 7 days.

2.2 SNP typing and recording of results

The SBE reaction and the post-extension treatment with SAP were performed as described in Ref. [1]. SAP-treated PCR fragments were prepared for capillary electrophoresis and denatured as described in Ref. [1] but separated and detected on various types of genetic analysers. Different separation polymers were used by the participants as indicated in Supplementary Table 1.

Different analysis software versions (Supplementary Table 1) were used for allele calling; however, all used a peak amplitude threshold of 50 relative fluorescence units (RFUs) and an allele balance cut-off value of 0.3. The organising laboratory provided appropriate panels and bin-settings that were adapted for the use of POP-4™ or POP-7™ polymer.

The results were to be returned in a provided excel sheet that recorded the result for each SNP in each sample (the revised Cambridge Reference Sequence (rCRS) [14] bases were specifically marked to facilitate the comparative analyses in part 2 of the exercise). The instructions for SNP profile interpretation included the following: "For each SNP, several results are possible: 1) one base is detected, the other base not (use yes and no in the excel sheet); 2) both bases are detected (use yes and yes per base and comment whether you have an opinion whether the sample represents a mixture or a heteroplasmy); 3) no base is detected (use no and no), which may be due to a nucleotide change at the primer binding site that is not covered by the degenerate bases in the primer, or low quality of the sample. Please note that due to the use of degenerate primers SNP r195 may show a broader peak and SNPs r16294, r182, 489 and 497 can show +1 trailing. The other SNPs do not show these effects." A remark column was present in the excel sheet for remarks on haplogroup type inference or observed inconsistencies that were encountered.

For the paper challenge, the participants were asked to indicate whether each of eight references could match ("use yes or no") any of the 13 samples typed by the participant. In a remark column comments like 'inconclusive' could be added. Reference data were provided for

the control region ranges 16024-16569 and 1-576 (these regions include all 18 SNP positions targeted by the mini-mtSNaPshot) in standard nomenclature (that mark deviations from the rCRS in the format 16216G, 16189Y, 309.1C or 523del). Finally, the laboratories were asked to return a questionnaire, the result sheet, the raw data and further comments to the organising laboratory.

3. Results and Discussion

*3.1. Part one – mini-mt*SNaPshot *typing and data analysis*

The 14 laboratories to which the samples were sent analysed 18 SNPs for 13 samples, which adds to a total of 3,276 SNP positions and 3,374 possible alleles as at seven SNP positions a bi-allelic call is expected. However by design, the mini-mtSNaPshot assay may fail SNP detection when a sample carries a mutation in the 3' part of the primer-binding site that is not covered by a degenerate base in the primer [1]. This was the case for nine SNP positions and when these are not considered 3,248 alleles remain for which effective genotyping can be determined. No incorrect allele calls were observed except in one multiplex extension reaction that appeared contaminated (and therefore regarded as not effectively genotyped). Five times the single-base extension of one of the multiplexes failed: four times for multiplex 2 and once for multiplex 1 (in four laboratories). This may be because multiplex 2 products were to be ExoSap-IT®-treated by the participants, while the organising laboratory treated the multiplex 1 products and checked SBE performance prior to shipment. Furthermore, 24 occasions of allele dropout were seen; eight times in multiplex 1 and 16 times in multiplex 2. Taken together, 3,170 of the 3,248 alleles (97.6%) were correctly genotyped. In the blank sample at two of the 350 positions a low drop-in peak was observed (both times 16311A), which is very low percentage. We infer that the technology is effective and can be readily applied in other laboratories.

The 13 samples can be grouped into five types: 1) single-source samples giving a full profile (four samples); 2) single-source samples with missing or low signals due to polymorphisms at primer binding sites not covered by the degenerate bases in the primers (four samples); 3) a clearly mixed sample with bi-allelic calls at multiple (five) loci; 4) samples with one bi-allelic signal (three samples) and 5) a mixed-up sample in which the PCR products for the two multiplexes did not originate from the same donor.

All four single-source samples (samples 1, 4, 5 and 6) were correctly typed by all participants. The four single-source samples with polymorphisms at the primer binding sites showed, as expected, missing or low signals (Table 1). The occurrence of dropout related to the proximity of the polymorphism to the 3' end of the primer: when the polymorphism corresponded to the -1 nucleotide (nt), -2 nt or -3 nt position in the primer (sample 10, position

16294; sample 9, position 16362; sample 3, position 182 respectively, Table 1) the targeted SNP was not detected by any of the participants, while with a polymorphism corresponding to the -16nt position in the primer (sample 9, position 16311, Table 1) all participants detected the targeted SNP. For the other SNPs, variable results were obtained (Table 1), which can be due to the different conditions in each laboratory (Supplementary Table 1).

Table 1
Four single-source samples, which show dropout or low peaks due to mutations at primer binding sites.

Sample	Affected SNPs[a]	Mutations at primer binding sites not covered by a degenerate base	Position relative to SNP (upstream)	Detected, often low signal	Dropout: no signal or below 0.3 cut-off
2	r182	195C[b], 198T and 204C	-13, -16 and -22nt	2/14	12/14
	16129	16114A	-15nt	7/13[c]	6/13[b]
3	r182	185T, 189G and 195C	-3, -7 and- 13nt	0/14	14/14
	16278	16270T and 16264T	-8 and -14nt	9/14	5/14
9	r16311	16327T	-16nt	13/13[b]	0/13[b]
	16362	16360T	-2nt	0/14	14/14
10	r16294	16295T	-1nt	0/14	14/14

[a] An 'r' before the position number indicates that the SNP is analysed using a reverse primer.
[b] A future primer may carry a degenerate base for 195 because of its known high mutability
[c] One laboratory failed typing one of the two multiplexes

One sample was a two-person mixture resulting in five bi-allelic calls in the mini-mtSNaPshot profiles. The mixture was based on equal nuclear DNA amounts for the two contributors, which will not necessarily result in a 1:1 mtDNA mixture. Nine of the fourteen laboratories detected all five bi-allelic calls (Table 2). Absence of the bi-allelic call occurred at two SNP positions: for position 185 the A allele remained five times below the allele balance cut-off value of 0.3 (the A to G ratio for these five instances varied from 0.14 to 0.25); for position 489 this happened four times to the C allele (and the C to T ratio for these four occasions varied from 0.25 to 0.28). Both alleles correspond to the same contributor that appears to have contributed less mtDNA notwithstanding equal nuclear DNA inputs. Nevertheless, all laboratories interpreted this sample as a clear mixture.

Table 2
Detection of the five bi-allelic positions in mixed sample 12.

5 bi-allelic positions	185 A/G	16294 T/C	16519 C/T	150 T/C	489 C/T
# laboratories detecting bi-allelic call	9/14	14/14	14/14	13/13[a]	9/13[a]

[a] One laboratory failed typing one of the two multiplexes

Three samples resulting in one bi-allelic signal were included in the exercise. Two of these samples were product of a two-person mixture (Figure 1B and 1E) and one sample was a single-source sample with a point heteroplasmy (Figure 1A). The detection of the bi-allelic signal depended a lot on the bases to be detected (Table 3): when two distinct bases were involved most participants identified this as a bi-allelic call; when the same base with a different sizing was to be detected, none of the participants indicated that the double peak had a bi-allelic nature although one laboratory noted a broad peak for this SNP position. The success of detecting the two different bases seems to depend on the balance between the two signals: the C/T signals for position 146 in sample 11 are of approximately similar strength and were detected by all participants (Table 3, Figure 1A); the A/G signals for position 185 in sample 7 are much less balanced (Figure 1B) and the lower A signal was not detected by two participants (Table 3). The A/A signal for position 73 in sample 13 derives from the combination of two samples that use a different specimen of the degenerate base carrying primer, as one sample has a C at position 72 and the other sample a T. Both examples were included in the exercise and were present amongst the single and full profile samples. Thus, the participants had the appropriate background to recognise that peak shifts may occur for position 73 (Figure 1C-E) and that an A/A peak can represent two distinct signals. Since the primer detecting position 73 has only one degenerate base that is at -1 nt position in the primer [1], the A/A signal is less likely from a single-source sample due to the binding of two primer forms. When the degenerate bases are further in the 5' part of the primer a double signal may occur. The presence of one bi-allelic call amongst the 18 analysed SNPs was interpreted as a point heteroplasmy by the participating laboratories (Table 3), which is understandable [21,22].

Table 3
Genotyping results for the three samples with one bi-allelic signal.

Sample	Type of sample	position	Bi-allelic signal	Interpretation
7	2-person mixture	185 A/G	12/14	Mostly regarded as heteroplasmy
11	Single-source, heteroplasmy	146 Y	13/13 [a]	Mostly regarded as heteroplasmy
13	2-person mixture	73 A/A	0/14	1 lab noted broader peak for 73A

[a] One laboratory failed typing one of the two multiplexes

The last sample type was a purposeful donor mix-up in that the PCR products for multiplex 1 derived from the donor used for sample 3 and those for multiplex 2 derived from the donor used for sample 9. Both these samples have primer binding site issues (Table 1) but since all affected SNPs reside in multiplex 1, only the primer binding site issues earlier seen for sample 3 (Table 1) are relevant for this mix-up sample 8, and these are dropout for position 182 (for sample 8 seen by 14/14 laboratories) and low signal for position 16278 (for sample 8 not

detected by 2/14 participants). The genotyping data will not reveal the mix-up; for that haplotype inference is needed as is described in the next section.

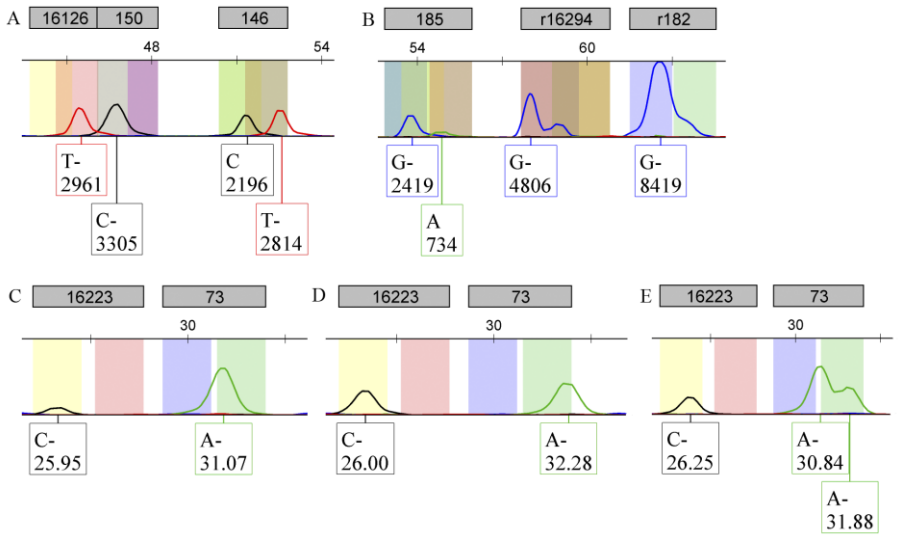

Fig. 1. Electropherograms showing mixtures and point heteroplasmy. Panel **A** illustrates a point heteroplasmy at position 146; Panel **B** a bi-allelic s at position 185 due to the presence of two donors. Panels **C** and **D** show an A peak at position 73 with a shifted location; **E** is a mixture of these two samples with an A/A peak at position 73. In panels A-B the allele call and peak height are given; in panels C-E the allele call and peak size are indicated.

3.2. Part one - Haplogroup inference

The participants were asked to provide comments such as haplogroup inference by their method of choice. Four of the 14 participants did not infer a haplogroup; the inferences of the other participants represent variable results (Table 4). Often it was not clear how haplogroup estimates were obtained, although one participant indicated to have used HaploGrep [23], a dedicated tool to determine haplogroup affiliation that employs the latest version of PhyloTree [24]. Alternatives are a manual PhyloTree check, MitoTool [25,26], the classifier tool implemented in the HmtDB database [27] or EMPOP (EDNAP Mitochondrial DNA Population Database, www.empop.org [28]), which is the platform most commonly used in forensics, that also gives the frequency of the haplotype. As EMPOP and HaploGrep were used by the participants, the haplogroup estimates derived from these platforms are presented in Table 4. It is worth mentioning that the two platforms indicate different haplogroups for samples 1, 5, 6, 7, 8, 9, 10, 11, 12 and 13 (Table 4, grey cells). This can be explained by the different databases that are used to estimate the haplogroups. While HaploGrep only uses the mutations listed in PhyloTree [23, 24], EMPOP takes the PhyloTree mutations and a curated database of approximately 25,000 mitogenomes into consideration [29]. The latter approach therefore involves all mutations of the haplotype in question including private mutations and those that are ignored in PhyloTree (e.g. positions 16519 and 523/524), which is especially relevant in the

case of (partial) control region sequences. Also, for multiple equally plausible haplogroup estimates EMPOP indicates the most recent common ancestor (MRCA), which is a coarser and more conservative approach. Still, the individual best matching haplogroup matches are listed in EMPOP with the associated haplogroup based on the full mitogenome information, and the user can trace back the MRCA estimate. From a practical standpoint, HaploGrep analyses are generally more prone to result in biased haplogroup estimates [30], which is less desired in forensic applications. When mini-mtSNaPshot profiles are used in these tools, the haplotypic information is based on at most 18 SNPs for EMPOP and 17 SNPs for HaploGrep (as position 16519 is ignored); when locus dropout occurs even less SNP positions can be used (and accordingly, these dropped out SNPs should not be entered in the input ranges for EMPOP and HaploGrep). Thus, one would expect a rather coarse haplogroup estimate and a very detailed estimate (Table 4, HaploGrep results in grey cells) may represent an overestimate and should be considered with caution.

Haplogroup inference may also be helpful for quality assessment. Both software are not designed for mixtures, such as sample 12 that is the two-person mixture with five bi-allelic sites among the 18 SNPs assessed. HaploGrep provides a warning; EMPOP returns a high frequency. Sample 8 is not a mixture but a purposeful mix-up for which we combined two donors of different descent: for multiplex 1 sample 3 (haplogroup L1b1a) and for multiplex 2 sample 9 (haplogroup D5a3) was used. HaploGrep provides the warning that there are four local private mutations associated with other haplogroups, which is a disquieting number considering that only 16 positions are regarded (drop out at position 182 and position 16519 is ignored in HaploGrep). Also EMPOP conveys suspicious results with this mix-up sample, as the haplogroup is inferred with one SNP missing and two SNPs as private mutations. None of the participants noted a possible mix-up, although one laboratory stated that there was not enough information to ensure the classification, and based the classification on only multiplex 1. Thus, sample mix-up may be recognised upon haplogroup inference, although this will be more difficult with samples of more similar haplogroups.

Evidently, when more genotyping information for a sample is entered into EMPOP or HaploGrep, either the same or a more detailed haplogroup estimate is expected; when an estimate in another branch is given, this may indicate that the estimate based on the 16-18 mini-mtSNaPshot SNPs is erroneous. To assess this aspect, the Sanger sequencing data for the HVS 1, 2 and 3 regions of eight single-source samples (samples 1 to 5 and 9 to 11) were submitted to EMPOP and HaploGrep and the haplogroup estimates were compared (Table 4). EMPOP reports for three samples the same haplogroup (samples 1, 2 and 4) and for five samples a more detailed haplogroup in the same branch (samples 3, 5, 9, 10 and 11). HaploGrep estimates three times the same haplogroup (samples 2, 4 and 10), returns once a more detailed

haplogroup in the same branch (sample 3) but provides a different branch for four samples (sample 1, 5, 9 and 11). These results illustrate the risks outlined above regarding haplogroup inference through HaploGrep with partial (control region) sequences.

3.3. Part two – Paper challenge

Next, a paper challenge was performed in which each mini-mtSNaPshot profile obtained in part 1 of the exercise was compared to eight reference mtDNA profiles described according to standard nomenclature [2]. This is an important step of the exercise as the mini-mtSNaPshot assay is regarded as a screening tool. Participants were asked to indicate whether a sample and reference profile could match, which is complicated by the different formats: the reference data name only bases deviant to the rCRS including deletions and insertions; the samples name the bases targeted by the SNaPshot assay. Table 5 shows an overview of the results of the paper challenge for each of the 13 samples. Five samples gave no matches to the reference profiles provided; the other eight samples matched with one or two reference samples. Overall, 93.2% of the comparisons were correct; 1.9% were incorrect and 4.9% were inconclusive. Incorrect scorings occurred more for some participants, for instance lab 13 appeared to match always at least one reference to a sample. Furthermore, some laboratories (3/14) tended to use inconclusive for matching reference profiles when only one non-rCRS SNP was present in the mini-mtSNaPshot profiles (samples 5 and 13, Table 5), which may be because the rCRS bases are more frequent and less discriminatory in the populations most encountered by the participants. In addition, some laboratories interpreted a SNP dropout as a deletion of the target base. This is incorrect, as a deletion will not generate a dropout in the mini-mtSNaPshot profile; instead the next base will be incorporated (consequently known deletion/insertion sites were not included in the mini-mtSNaPshot assay). Actually, when a mini-mtSNaPshot dropout occurs, this is information about the nucleotides at the primer-binding site. Mixed samples can match more references, as is seen for sample 12. Samples with one bi-allelic signal may be regarded as a two-component mixture or as single-source with a heteroplasmic position. Such a heteroplasmy does not need to be present in all tissues. This is illustrated by sample 7, which is a two-person mixture with one bi-allelic call for which one of the people was included as a reference profile. Ten of the 14 laboratories matched the sample to the reference profile (Table 6), and assumed a tissue effect. The other four laboratories concluded ‘inconclusive’ as the point heteroplasmy present in sample was not present in the reference profile.

Table 4
Results of haplogroup inference by EMPOP, HaploGrep (using PhyloTree build 17) and the participants.

Type	Sample	Locus dropout[a]	Polymorphisms	EMPOP v3/R11		HaploGrep[b]		Participants[c]	Sanger data[d]	
				Frequency	MRCA	Haplogroup	Warnings & errors		EMPOP	HaploGrep
single-source, no primer binding issues	1	none	73G 150T 16126C 16294T 16519C	62/27242	T2e1	T1a10a	ambiguous best results	6x T; 1x T [private 150T]; 2x T2; 1x T2e; 4x no inference	T2e1	T2e1
	4	none	146C 152C 195C 16362C	9/27242	H8+(114)	H8+(114)+152	ambiguous best results	1x H; 1x HV; 1x H/V/HV; 4x H8; 1x H8b; 1x H8/H8c/H31; 5x no inference	H8+(114)	H8+(114)+152
	5	none	16519C	1760/27242	H/R0	H2a2a1	ambiguous best results; low quality	1x H2a2a1; 1x H; 1x H/V/HV; 1x H/HV; 2x R0: HV/H/H2a; 1x R0; 7x no inference	V3c	V3c
	6	none	73G 195C 497T 16311C 16519C	96/27242	K1a	K1a4a1a+195	ambiguous best results	1x R; 1x R*(not J/T)/U; 1x H11/R1/R8/K1a; 1x K; 5x K1a; 1x K1a4a1a; 4x no inference		
single-source, with primer binding issues	2	none	73G 150T 152C 182T 195C 16129A 16223T 16278T 16362C	64/27242	L2b1a	L2b1a	ambiguous best results	1x L; 1x L2 (possibly L3/N); 1x L2a'b'c'd/L2b'c'd; 1x L2b*; 2x L2b1a; 1x L3b1a; 1x X2; 1x H1/H3/H4/H12/H13/H14/H44; 5x no inference	L2b1a	L2b1a
	2	182, 16129	73G 150T 152C 195C 16223T 16278T 16362C	70/27242	L2b1a	L2b1a	ambiguous best results			
	3	182	73G 152C 185T 195C 16126C 16223T 16270T 16278T 16311C 16519C	336/27242	L1b	L1b	ambiguous best results	1x L; 1x L1; 4x L1b; 1x L1b1a18; 1x L3h1a2b; 1x L2'3'4'6/ L3'4'6; 1x U6*; 4x no inference	L1b1a	L1b1a1+189
	3	182, 16278	73G 152C 185T 195C 16126C 16223T 16270T 16311C 16519C	337/27242	L1b	L1b	ambiguous best results			
	9	16362	73G 150T 489C 16223T	133/27242	M (mostly D5/M7b4)	M7b1a1b	ambiguous best results	4x M; 1x M/L3; 1x M7b1; 1x M62'68; 1x D; 1x D4t [private150T]; 5x no inference	D5a3	D5a3
	10	16294	73G 146C 489C 16223T 16362C 16519C	80/27242	M (mostly D4b2b1/M7c3c)	M7c1a4a	ambiguous best results	4x M; 2x D; 1x D4*; 1x D4j6/D4t; 1x D4t [private 146C]; 1x C/D/E/G/M/Q/Z; 4x no inference	M7c1	M7c1a4a
clear mixture	12	none	73G 150Y 489Y 185R 16126C 16294Y 16519Y	1191/27242	JT	J1c1c	5 global private mutations; misses 3 expected SNPs; 5 heteroplasmic positions	1x JT; 1x R0/HV/H/H2a; 12x no inference		
one biallelic signal 2-person	7	none	73G 489C 185R 16126C	427/27242	J1c/J1b; ignored mutation G/A185R	J1b1a1d	1 heteroplasmic position: 185R	2x JT; 3x J; 1x J1; 1x J1c*; 1x M; 1x M3[no 16223T]; 5x no inference		
	13	none	16519C[e]	1760/27242	H/R0	H2a2a1	ambiguous best results; low quality	1x H2a2a1; 1x H; 1x R0/HV/H/H2a; 1x H/V/HV; 1x H/HV; 2x R0; 7x no inference		
1-ps	11	none	73G 146Y 152C 16129A 16223T 16519C	70/27242	I2a/I3a; ignored mutation C/T146Y	A7	1 heteroplasmic position: 146Y	1x H mp1 only/ inconclusive; 2x N; 1x I (but rare in N and L3); 1x L3/N*/ I; 2x L3c'd/L3d1b3; 1x D4a[no 489C, 16362C]; 6x no inference	I3a	I3a
mix-up	8	182	73G 150T 185T 195C 489C 16223T 16270T 16278T 16311C 16519C	0/27242	**L1b1a;** missing T152C; private mutations C150T T489C	M2a1b	4 local private mutations associated to other Hg's; 4 local private mutations - 3 in L1b3; moderate	1x N1*; 1x L/M; 3x M; 1x L1b1a; 1x M51b1[private 185T/195C/16270T]; 1x M51b1; 1xL3h1a2b/M51b1/M43A/M29'Q/M57/M62'68/D4k; 5x no inference		

quality

[a] The input range is 73 146 150 152 182 185 195 489 497 16126 16129 16223 16270 16278 16294 16311 16362 16519. Loci that drop out (see Table 2) were omitted from the input range.
[b] Haplogroup cells are coloured grey when the inference deviates from the MRCA warnings & error cells are coloured according to the quality colour flags generated in HaploGrep.
[c] One participant used HaploGrep: these results are indicated in blue.
[d] The Sanger sequencing data comprise HVS 1, 2 and 3, in EMPOP InDels in the control region are not disregarded.
[e] The bi-allelic signal is a double A signal at position 73, which cannot be entered as such.

Table 5
Results of the paper challenge.

Type	Sample	Status	Score for # comparisons			Remark
			Correct	Incorrect	Inconclusive	
Single-source, no primer binding issues	1	no match: 7 refs	98	0	0	
		match: 1 ref	14	0	0	
	4	no match: 8 refs	102	2	8	wrongful match lab13
		match: 0 refs	0	0	0	
	5	no match: 6 refs	78	0	6	wrongful no match lab11 & lab12; labs scored inconclusive as only one variant could be compared with reference data
		match: 2 refs	18	4	6	
	6	no match: 8 refs	111	1	0	wrongful match lab13
		match: 0 refs	0	0	0	
Single-source, with primer binding issues	2	no match: 8 refs	103	1	8	wrongful match lab13; one lab inconclusive for all refs due to failure multiplex 2
		match: 0 refs	0	0	0	
	3	no match: 7 refs	96	2	0	lab11 & lab12 wrongful match; one lab scored inconclusive due to presences of 182del and 16278del
		match: 1 ref	11	2	1	
	9	no match: 7 refs	96	0	2	wrongful no match lab12
		match: 1 ref	11	1	2	
	10	no match: 7 refs	98	0	0	wrongful no match lab12
		match: 1 ref	12	1	1	
Clear mixture	12	no match: 6 refs	72	0	12	labs scored inconclusive or no match because sample represents mixture. That not always all five bi-allelic calls were detected (Table 2), effected the outcomes.
		match: 2 refs	16	4	8	
1 bi-allelic signal 2-ps	7	no match: 7 refs	98	0	0	heteroplasmic position (185R) present in sample, reference 6 has 185A
		match: 1 ref	10	0	4	
	13	no match: 6 refs	78	0	6	wrongful no match lab11 & lab12; labs scored inconclusive as only one variant could be compared with reference data
		match: 2 refs	18	4	6	
1 bi-allelic signal 1-ps	11	no match: 8 refs	107	3	2	wrongful match lab11, lab12 & lab13
		match: 0 refs	0	0	0	
mix-up	8	no match: 8 refs	110	2	0	wrongful match lab10 & lab13; lab 10 interpreted mixture (due to contamination)
		match: 0 refs	0	0	0	

4. Concluding remarks

A collaborative exercise was organised amongst 15 laboratories on an mtDNA screening assay that targets 18 control region SNP positions. The overall success rate in obtaining mini-mtSNaPshot results was high indicating that the method is stable and reproducible. Challenging samples were included such as a double 73AA peak representing a true bi-allelic signal due to the presence of two donors and a purposeful mix-up that comprised the PCR products for multiplex 1 and multiplex 2 from different donors of distinct phylogenetic descent. None of the participants noted this mix-up, although haplogroup inference by EMPOP and the HaploGrep tool would have come with a warning indicating a strikingly high number of private mutations when considering the number of positions analysed. This warning, however, may also appear

with other samples, e.g. unique single-source haplotypes that are distant to neighbouring haplotypes by multiple private mutations. In casework practice, this mixed-up sample would (most likely) be selected for analysis by sequencing (as it represents a deviant profile) upon which the mix-up would have been noted (as the amplicons in set 1 and set 2 are partly overlapping). Nevertheless, we advise to apply phylogenetic checks for contamination, mix-up and phantom mutations also when assessing the 18 control region positions.

When participants were requested to compare the mini-mtSNaPshot results to reference mtDNA profiles presented in standard nomenclature, some laboratories generated more incorrect results than others and we infer that training and guidelines may be helpful. This paper challenge also showed that the set of 18 SNPs is useful to screen a set of references and this may stimulate the expansion of such screening methods in forensic laboratories. This would increase efficiency in forensic casework not only when samples are analysed through Sanger sequencing but also when MPS is applied. As a small additional study, four of the participants analysed sample 11 and another total DNA sample through various MPS approaches for which the results are described in Supplementary Text 1, Supplementary Table 2 and Supplementary Figure 1.

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